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FORMULATION OPTIMIZATION OF A HYDROCOLLOID DRESSING

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ABSTRACT

A methodology of mixture experiments has been applied to the formulation of a multicomponent hydrocolloid dressing. Using an extreme vertices statistical design, a semi-occlusive dressing composed of dextran, phospholipid, glycerol and sodium lauryl sulphate has been formulated, which checks evaporative water loss (EWL) from the excised wound surface of rats to an optimum level.

INTRODUCTION

Extensive skin loss following burn injury poses a serious problem and results in increased evaporative water loss (EWL)[1] and intense catabolism[2]. Various burn dressings[3,4,5,6] have been used in these situations and one of the main objectives of these dressings is to control EWL and to provide a moist environment at the wound site for rapid epithelialization[7,8,9]. An optimum level of occlusion is desirable because high EWL will dehydrate the skin to form a thick scab while low EWL will cause exudate build up on the wound surface leading to the danger of infection[10]. It is the purpose of this study to develop a semi-occlusive, flexible hydrocolloid

dressing, which when applied onto the wound surface, will provide a moist environment but avoid exudate accumulation. The dresssing is composed of dextran, L-α-phosphatidylcholine, glycerol and sodium lauryl sulphate. A statistical optimization technique has been employed to locate the optimum formulation of the dressing with the four components.

Development of a pharmaceutical formulation possessing all the desirable properties is a complex and difficult task. Conventionally, empirical approaches are used which in general involve variation of one factor at a time whilst other factors are kept at fixed values. These methods are time consuming, costly, unreliable and are acceptable when the factors are independent to one another[11]. Interactions between the factors are not considered and different formulations may be obtained if the order of investigation is altered.

Various statistical optimization techniques such as factorial design[12-18], central composite design[19-22] and simplex lattice design[23-24] have been widely employed to increase the efficiency of formulation development works. However, these designs can not be applied when it is desired to evaluate the effect of each ingredient on the physico-chemical properties of a formulation and also do not take into account those situations when the components are associated with lower and upper constraints. An extreme vertices design[25,26] can be used in such situations and has been utilized successfully in preformulation studies[27,28]. The independent variables in this design are the ingredients quantities of the formulation. In this design, each component is restricted by the lower (L_i) and upper (U_i) bounds imposed on the system, due to the physical or chemical limitations of the formulation or by selection of the concentration range by the experimenter.

The constraint region can be expressed as:

$$0 \le L_i \le X_i \le U_i \le 1 \qquad i= 1, 2, 3, ..., q \tag{1}$$

and

$$\sum_{i=1}^{q} X_i = 1 \tag{2}$$

where

q = number of components in the mixture

X_i = proportion of component i, and
L_i and U_i = constraints on X_i

Here the sum of proportions of all the components is always one. Hence the change in any one component is compensated by changing the proportions of the remaining components. In addition, each proportion is within its specified limits. Due to all these restrictions, the experimental region of this design is a hyper-polyhedron (q-1 dimensions). The construction and shape of the compositional region in the hyperpolyhedron is defined by equations (1) and (2). The basic design consists of all the vertices of this hyper-polyhedron. If necessary, edge centroids, centroids of the faces and overall centroid can be added to estimate the experimental error and to estimate all the coefficients of the response model.

The number of possible vertices which can be generated for a particular system are given by $q.\ 2^{q-1}$, where q are the number of variables defined in equation 1 and 2. However, only those vertices where the component level falls within the specified range i.e., between L_i and U_i for each component, are used as experimental points.

Initial design points usually involve all the vertices and the overall centroid. The overall centroid is included to estimate the curvature. Scheffe's second order polynomial model[29] and Becker's models[26] are commonly used in the analysis of the design points data[26,30-32]. Using these models, a correlation is predicted between the components of the mixture and the dependent variables. After analysing these data points, if necessary, face centroid and edge centroids can also be included into the design to improve the accuracy of the model.

EXPERIMENTAL

Materials

Clinical grade dextran of molecular weight 81,500 and L- α -phosphatidylcholine from fresh turkey egg yolk were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Sodium lauryl sulphate, specially pure, was purchased from BDH, Poole, UK. Glycerol of BP grade was procured from Pharmaceutical Sales and Marketing Ltd., Auckland, NZ. Drierite®, the desiccant was obtained from W. A. Hammond Drierite Co., Xenia, Ohio, USA.

Instruments

Ultrasonic probe (Branson Ultrasonic Corp., Conn, USA) was used to solubilize the phospholipid. Water was removed from the casting solution using a vacuum oven (Precision Scientific Co., USA). Glass rings of 70mm in diameter with unsintered Teflon® film or Whatman® sealing film securely fastened on one side using a rubber band, were used for casting the membranes. Rotameter (Platon® GA Platon Ltd., Basingstoke, UK) was used to control the flow of compressed air.

Ventilated Hygrometer Chamber System to Study Evaporative Water Loss of Hydrocolloid Dressings

The instrument used in this study was similar to that described by Wang et al.[33]. The apparatus consisted of a flowmeter, a manometer and a hemispherical open-bottomed glass hygrometer chamber with outer diameter of 2.6 cm and thickness of 0.3 cm (cross-sectional area equal to 3.14 cm^2) and an U-tube containing approximately 20g of desiccant. Dry compressed air with inlet pressure of 20mm Hg and a flow rate of 1L/minute, was used as a carrier gas. The absorption of moisture by the U-tube from the atmosphere was checked by a guard tube filled with the desiccant.

METHODS

Determination of Design Points in the Formulation of Hydrocolloid Dressing

Concentration range for each component i.e., dextran (65-90% w/w), phospholipid (0-20% w/w), sodium lauryl sulphate (0.5-5% w/w) and glycerol (0-34.5% w/w) was initially selected (see Table 1). A total of 32 vertices were then generated and from these only 10 vertices (indicated by asterisks in Table 1), where all components add up to unity and the level of each component fell within the specified range, were selected as the design points. The overall centroid was calculated by averaging all the factor level of all the components. Therefore, a total of eleven formulations of hydrocolloid dressings, using ten vertices and the overall centroid as the design points, were initially prepared and evaluated.

Preparation of Hydrocolloid Dressings

Eleven formulations were prepared in this study according to the following procedure: 26-36g of dextran was dissolved in 150ml of distilled water at room

TABLE 1
Design points for the given four components system

	Components and range			
	Dextran (D) (65 - 90%)	Phospholipid (L) (0 - 20%)	Sodium lauryl sulphate (S) (0.5 - 5%)	Glycerol (G) (0 - 34.5%)
Possible vertices				
1*	65.0	0.0	0.5	34.5
2*	65.0	0.0	5.0	30.0
3*	65.0	20.0	0.5	14.5
4*	65.0	20.0	5.0	10.0
5*	90.0	0.0	0.5	9.5
6*	90.0	0.0	5.0	5.0
7	90.0	20.0	0.5	- a
8	90.0	20.0	5.0	- a
9	65.0	34.5 b	0.5	0.0
10	65.0	0.0	0.5	34.5 c
11	65.0	30.0 b	5.0	0.0
12	65.0	-	5.0	34.5 a
13*	90.0	9.5	0.5	0.0
14	90.0	-	0.5	34.5 a
15*	90.0	5.0	5.0	0.0
16	90.0	-	5.0	34.5 a
17	65.0	0.0	35.0 b	0.0
18	65.0	0.0	0.5	34.5 c
19	65.0	20.0	15.0 b	0.0
20	65.0	20.0	-	34.5 a
21	90.0	0.0	10.0 b	0.0
22	90.0	0.0	-	34.5 a
23	90.0	20.0	-	0.0 a
24	90.0	20.0	-	34.5 a
25	99.5 b	0.0	0.5	0.0
26	65.0	0.0	0.5	34.5 c
27	95.0 b	0.0	5.0	0.0
28	60.5 b	0.0	5.0	34.5
29*	79.5	20.0	0.5	0.0
30	45.0 b	20.0	0.5	34.5
31*	75.0	20.0	5.0	0.0
32	40.5 b	20.0	5.0	34.5
Overall centroid				
33*	77.45	9.45	2.75	10.35

a values could not be calculated as sum of other components was >100%

b the amount calculated was outside the specified range

c repeat points

* vertices where the components level were within the specified range

temperature. 0-8g of L- α- phosphatidylcholine was solubilized in another 150ml of distilled water at 70°C containing 0.2-2g sodium lauryl sulphate, with the aid of ultrasonification for two minutes at a 125W setting. The above two solutions were mixed and 0-13.8g of glycerol was added. The resulting mixture was then sonicated in an ultrasonic bath for five minutes and poured into eight membrane casting rings. The rings were then maintained at 50°C at a reduced pressure of 15cm mercury for five hours in a vacuum oven. Once dried, the membranes were removed from the assembly by unfastening the base film. All membranes prepared were stored at 40% RH to prevent them from desiccation.

Evaporative Water Loss Measurements using the Ventilated Hygrometer System

The effectiveness of the dressings in controlling evaporative water loss from the wound surface was evaluated using an excised rat model. Sprague-Dawley rats, weighing 200-250g, were used for the study. The animals were anaesthetized by an intraperitoneal injection of Pentobarbital (Nembutal®, 40mg/Kg) and their dorsal surfaces shaved with a # 40 blade electric clipper (Thrive®, Daito Electric Machine Ind. Co. Ltd., Japan). Reese Drum Dermatome was used for creating split-thickness injury. An area of 2 x 2cm was mapped out on the dorsal shaved surface and coated with special glue which adhered to the drum of the dermatome. The area to be harvested was pulled up against the drum and sliced off at a preset thickness with a scalpel blade, which was manually moved back and forth across the drum face. After injury, blood was soaked up using a gauze pad. The membrane was then fixed onto the bottom of the hygrometer chamber with instant glue to provide an air tight seal around the rim of the chamber. The assembly was secured over the wound surface with glue applied on the edges of the created wound.

All experimental procedures were performed according to the rules set by the University of Otago Committee on Ethics in the Care and Use of Laboratory Animals.

Water evaporated from the wound surface, with or without the dressings, was removed by compressed air and collected in the U-tube containing Drierite®. The quantity of water loss from the wound surface during the experiment was determined by the weight gain of the desiccant ($W_{Final} - W_{Initial}$).

The EWL was calculated using the following equation:

$$EWL\ (g/m^2/24h) = \frac{W_{Final} - W_{Initial}}{\text{Area of the wound } (m^2) \times \text{Time } (24h)}$$

The area of the wound was measured using a calliper. The EWL for each dressing was measured four times. Each experiment was carried out for four hours. All the animals during experimentation were kept and maintained at body temperature of 35°C.

Analysis of Data

The data obtained were analysed using SAS computer package[34]. Different polynomial models were fitted to the data. Polynomial equations and contour plots were generated to establish the correlation between the dependent variable (e.g., EWL) and the independent variables (i.e., each component of the hydrocolloid dressing) as well as their interactions.

RESULTS AND DISCUSSION

All the eleven formulations, prepared in this investigation, were transparent. The weight per membrane was about 5g. Hydrocolloid dressings without glycerol were found to be brittle when dried. All the formulations were observed to have good adhesion properties on the excised wound surface. The EWL data obtained from the intact skin, uncovered excised wound and wound covered with various dressings, using ventilated hygrometer chamber, are presented in Table 2.

Initially, Scheffe's quadratic model of the form :

$$EWL\ (Y) = \sum_{i=1}^{q} B_i X_i + B_{ij}\ X_i X_j \qquad (3)$$

where

Y = dependent variable

q = number of variables

X_i and X_j = independent variables

B_i and B_{ij} = regression coefficients associated with the variables

was used to fit the EWL data. The following regression equation with a R^2 value of 0.999 was obtained.

$$EWL = 17.04\ (D) + 384.0\ (L) - 11479.2\ (S) + 168.0\ (G) - 535.2\ (D\ L) + 12050.4\ (D\ S) - 268.8\ (D\ G) + 12072.0\ (L\ S) - 376.8\ (L\ G) + 12220.8\ (S\ G) \qquad (4)$$

where D, L, S and G represent the concentration (% w/w) of dextran, phospholipid, sodium lauryl sulphate and glycerol in the formulation respectively.

TABLE 2
EWL of Various Formulations of Hydrocolloid Dressings

Formulation Type	EWL ($g/m^2/24h$)[a] (Mean ± SD)[b]
1	11872 ± 2176
2	11145 ± 1766
3	8923 ± 1785
4	10356 ± 1039
5	10250 ± 3648
6	9739 ± 276
7	8181 ± 907
8	9350 ± 2664
9	7908 ± 199
10	7596 ± 1015
11	9621 ± 2200

a calculated after four hours

b mean of four experiments

The high R^2 of this model may indicate that this model fits the data very well. However, a closer examination of its predicted power indicated otherwise. The model provided a F-value of 175.13 and root mean square error (MSE) of 0.0032. A formulation consisting of 75% w/w dextran, 10% w/w of phospholipid, 10% w/w of glycerol and 5% w/w of sodium lauryl sulphate, was predicted by the model, to provide maximum protection against EWL. The EWL predicted for this composition was 3120 $g/m^2/24h$, which is well below the values obtained experimentally (see Table 2). To overcome the shortcomings of this polynomial model, the following Becker's models were used as alternatives:

$$\text{H1:} \quad E(Y) = \sum B_i X_i + \sum B_{ij} \min (X_i, X_j) \tag{5}$$

$$\text{H2:} \quad E(Y) = \sum B_i X_i + \sum B_{ij} X_i X_j / (X_i + X_j) \tag{6}$$

$$\text{H3:} \quad E(Y) = \sum B_i X_i + \sum B_{ij} (X_i X_j)^{1/2} \tag{7}$$

Like Scheffe's quadratic model, both H1 and H3 models did not fit the data. However, H2 model provided a better fit to the data ($R^2 = 0.999$) with a F-value of 486.35 compared to 271.76 and 253.69 and a MSE of 0.0019 compared to 0.0030 and 0.0026 for H1 and H3 respectively. The fitted H2 model was:

$$EWL = 12.10\,(D) + 132.48\,(L) - 1029.36\,(S) + 42.72\,(G) - 180.48\,(DL/D + L) + 1051.92\,(D\,S/D + S) - 48.72\,(D\,G/D + G) + 72.0\,(L\,S/L + S) + 13.44\,(L\,G/L + G) + 53.28\,(S\,G/S + G) \quad (8)$$

Using equation 8, a predicted optimum formulation of HSS, composed of 75% w/w of dextran, 10% w/w of phospholipid, 5% w/w sodium lauryl sulphate and 14.5% w/w of glycerol with an EWL of 8116.8 $g/m^2/24h$ was selected. In order to check the predictive power of this model, hydrocolloid dressings were prepared using the optimized formulation and evaluated for their EWL. An experimental EWL of 8283.3 $g/m^2/24h$ was obtained which demonstrates an excellent agreement with the predicted value.

To examine the effects of various components on the EWL, contour maps were constructed. Figures 1 to 3 are the truncated contour plots of EWL at fixed concentrations, namely 0.5, 2.75 and 5% w/w of SLS respectively. The amount of glycerol at any point can be calculated due to the unity constraint imposed on the system. The truncated shape of the plots is due to the restriction imposed on different components of the dressings.

Results presented in Figures 1 to 3 also reveal that the concentration of dextran and glycerol of the membranes have minimal effect on the occlusiveness of the hydrocolloid dressings. On the other hand, the amount of L- α - phosphatidylcholine in the dressings has a marked effect on the EWL from the wound surface. Membranes with higher concentrations of phospholipids have lower EWL. An increase in the phospholipid content from 0 to 10%w/w resulted in a decrease of EWL from 11760 to 8160 $g/m^2/24h$. Increase of the phospholipid content from 10 to 20%w/w, however, did not provide any significant reduction in EWL. Sodium lauryl sulphate was found to counteract the effect of phospholipid on the occlusiveness of the dressings. At a fixed concentration of phospholipid of 10%w/w, an increase in SLS from 0.5 to 5% w/w caused an increase of EWL from 7920 to 9120 $g/m^2/24h$. These results

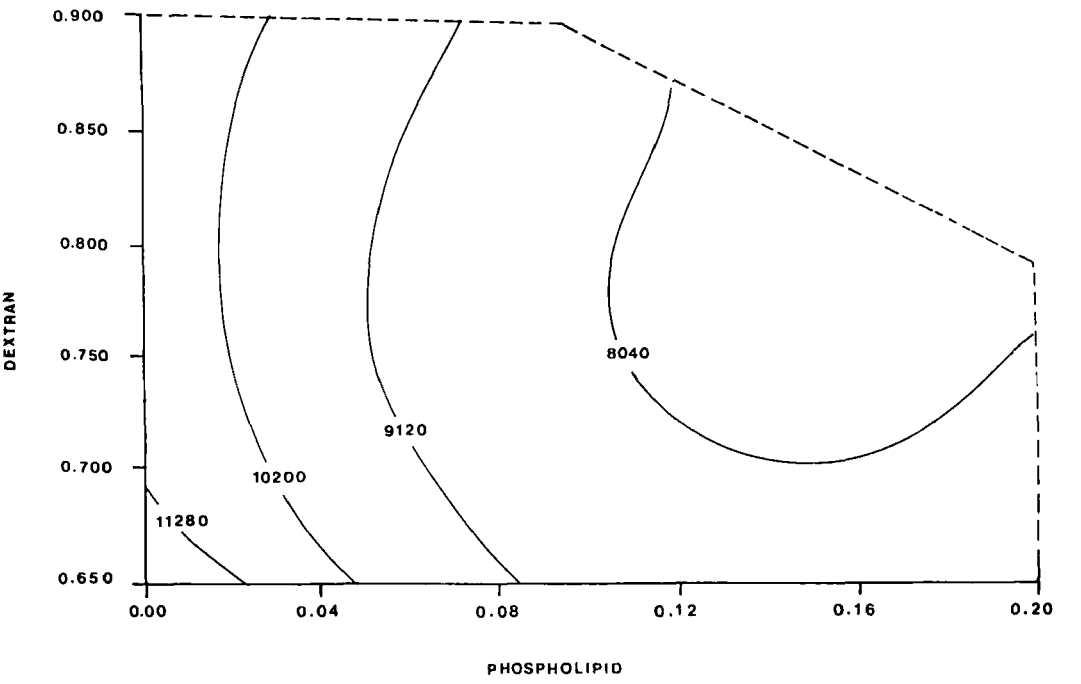

FIGURE 1
Contour plot showing the effect of different concentrations of dextran and phospholipid on EWL ($g/m^2/24h$) at 0.5%w/w of sodium lauryl sulphate in the hydrocolloid dressing.

indicate that the optimized hydrocolloid dressing with an EWL of 8283 $g/m^2/24h$, slightly more than half of that of the freshly-excised wound (13392 ± 273 $g/m^2/24h$), would provide the desired occlusiveness at the wound surface[35].

Dextran is selected as the hydrocolloid component of the dressing because dextran is a nontoxic, nonantigenic polysaccharide and is widely used as a plasma expander to resuscitate the burned patients[36]. Clinical grade dextran of molecular weight of 81,500 is employed, as higher molecular weight dextran has been shown to be antigenic[37]. A concentration range of 65-90% w/w of dextran in the formulation was selected, as membranes with concentration less than 65% w/w were too sticky to remove from the membrane casting apparatus, while those prepared with higher concentrations (>90% w/w) were glassy films and fragile in nature.

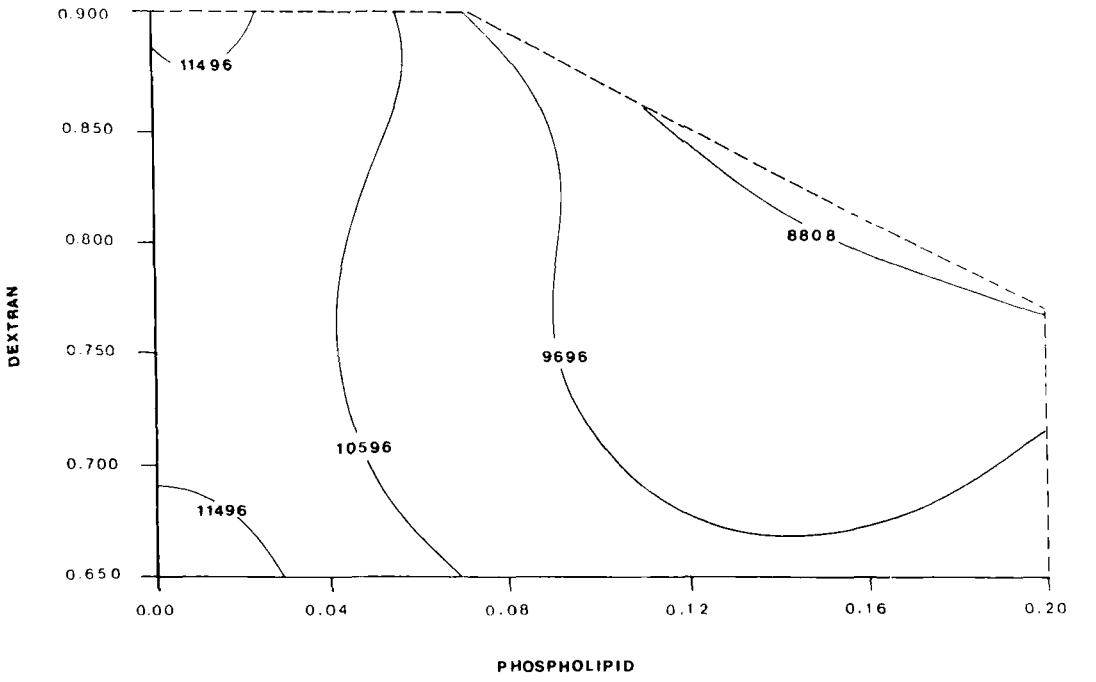

FIGURE 2
Contour plot showing the effect of different concentrations of dextran and phospholipid on EWL ($g/m^2/24h$) at 2.75%w/w of sodium lauryl sulphate in the hydrocolloid dressing.

It has been demonstrated that barrier functions of the skin on evaporative water loss, are primarily due to the presence of various lipid components i.e., cholesterol, glycolipids, phospholipids and free fatty acids[38-40]. Removal of these lipids from the skin results in an excess EWL[41,42]. A similar loss of lipids has also been reported in patients with thermal injuries[43]. Topical application of lipids such as free fatty acids and their esters has been shown to reduce the water loss from the wound surface and promote healing[44-46]. In this study, phospholipid was used to provide some occlusiveness to the dressings. The normal skin lipid content has been found[47] to vary between 10 - 15% w/w . Therefore, the effect of the phospholipid component in controlling EWL from the wound surface was investigated by varying its concentration from 0 to 20%w/w of the hydrocolloid dressings.

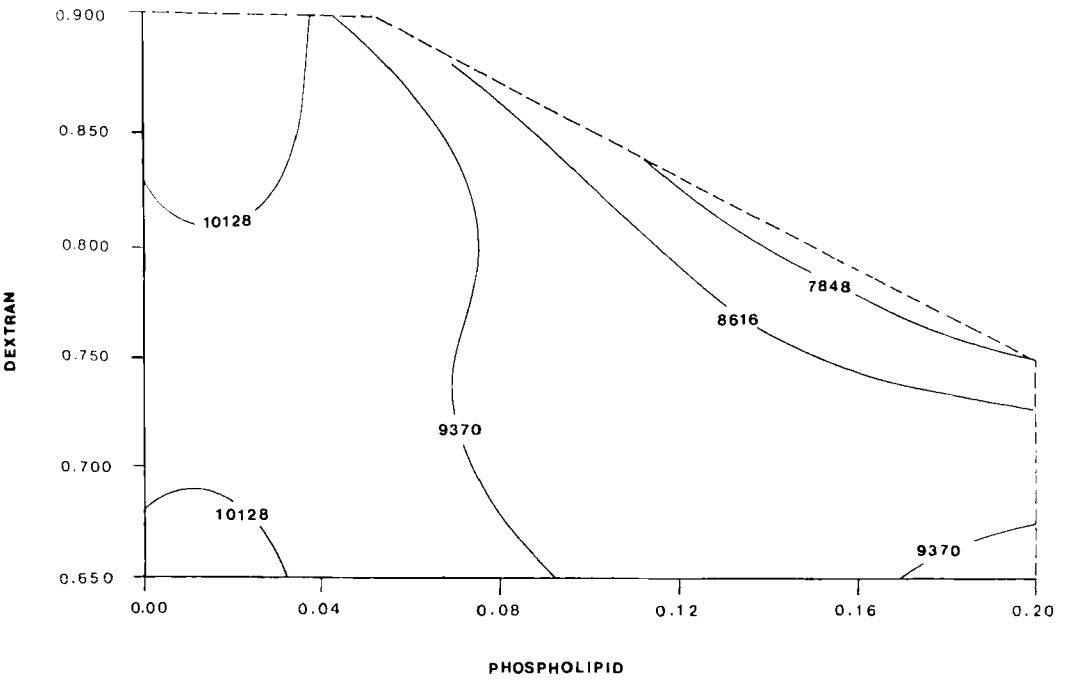

FIGURE 3
Contour plot showing the effect of different concentrations of dextran and phospholipid on EWL ($g/m^2/24h$) at 5%w/w of sodium lauryl sulphate in the hydrocolloid dressing.

In a preliminary study, anionic (sodium lauryl sulphate), cationic (cetyl trimethylammonium bromide) and non-ionic surfactants (tween 85 and span 85) were investigated for their ability to solubilize phospholipid in the formulation. Of all the surfactants investigated, only sodium lauryl sulphate was found to be effective in solubilizing phospholipid uniformally into the mixture and to provide transparent membranes. Hence sodium lauryl sulphate was used in the formulation of the dressings. 0.5% w/w of sodium lauryl sulphate was required in the solubilization process. Membranes made with sodium lauryl sulphate <0.5% w/w were patchy and turbid in appearance.

Glycerol, a hydrophilic plasticizer, is used to provide flexibility and elasticity to the dressings. Initially, it was also observed that hydrocolloid membranes with

glycerol concentration higher than 40% w/w were too sticky to remove from the membrane casting apparatus. Therefore, glycerol concentration in the range of 0-34.5% w/w was selected.

The extent of the created injury resembles deep partial-thickness burns. The experiment was conducted for only four hours as this was the maximum time that the animals could be kept under anaesthesia. The air flow rate within the collecting chamber during the experiment was maintained at 1L/minute. It has been demonstrated that a high flow rate would reduce EWL due to the cooling effect on the skin[48] and the pressure build up within the chamber. The average EWL through the intact skin was 1238 ± 350 g/m^2/24h (at 35°C body temperature) which is approximately twice the EWL (624 ± 168 g/m^2/24h) observed by other investigators[33]. This difference in EWL is probably due to the higher flow rate (2L/minute) used by Wang et al.[33].

In conclusion, a semi-occlusive, flexible hydrocolloid dressing has been obtained which checks EWL from the excised wound surface to an optimum level. The optimized dressing, with its ability to maintain a moist environment at the repairing wound site, would promote wound healing. This study also indicates that by incorporating a hydrophobic compound, the EWL of a hydrocolloid dressing can be altered. Such findings are important as they provide an alternative to promote occlusion of a dressing without the use of impermeable polymeric film. An semi-occlusive dressing with an optimum EWL can be fabricated using such an approach.

In addition, this study demonstrates the importance of extreme vertices design in the formulation of a multicomponent hydrocolloid dressing. Using this technique, the number of experiments carried out were reduced considerably. Although only the effect of each component on the EWL has been investigated, extreme vertices design can also be employed for the simultaneous optimization of other desirable properties.

REFERENCES

1. C. Jelenko, Ann. Surg., 165, 83 (1967).
2. J. W. L. Davies, "Physiological Responses to Burning Injury", Academic Press, London, 1982.

3. G. B. Park, Biomat. Med. Dev. Art. Org., 6, 1 (1978).
4. M. J. Tavis, J. W. Thornton, R. T. Danet and R. H. Bartlett, Surg. Clin. North Am., 58, 1233 (1978).
5. J. C. Lawrence, Injury, 13, 500 (1982).
6. K. J. Quinn, J. M. Courtney, J. H. Evans, J. D. S. Gaylor and W. H. Reid, Biomaterials, 6, 369 (1985).
7. G. D. Winter, Nature, 193, 293 (1962).
8. C. D. Hinman and H. I. Maibach, Nature, 200, 377 (1963).
9. A. Barnett, R. L. Berkowitz, R. Mills and L. M. Vistnes, Am. J. Surg., 145, 379 (1983).
10. A. B. Barnett, B. Dave and L. M. Ksander, J. Surg. Res., 41, 326 (1986).
11. J. M. Gallo, C. T. Hung and D. G. Perrier, Int. J. Pharm., 22, 63 (1984).
12. D. E. Fonner, J. R. Buck and G. S. Banker, J. Pharm. Sci., 59, 1587 (1970).
13. J. B. Schwartz, J. R. Flamholz and R. H. Press, J. Pharm. Sci., 62, 1165 (1973).
14. S. Dincer and S. Ozdurmus, J. Pharm. Sci., 66, 1070 (1977).
15. J. B. Schwartz, J. Soc. Cosmet. Chem., 32, 287 (1981).
16. E. Fenyvesi, K. Takayama, J. Szejtli and T. Nagai, Chem. Pharm. Bull., 32, 670 (1984).
17. M. R. Harris, J. B. Schwartz and J. W. McGinity, Drug Dev. Ind. Pharm., 11, 1089 (1985).
18. C. F. Dick, R. A. Klassen and G. E. Amidon, Int. J. Pharm., 38, 23 (1987).
19. E. Shek, M. Ghani and R. E. Jones, J. Pharm. Sci. 69, 1135 (1980).
20. K. Takayama, N. Nambu and T. Nagai, Chem. Pharm. Bull., 31, 4496 (1983).
21. Y. Akitoshi, K. Takayama, Y. Machida and T. Nagai, Chem. Pharm. Bull., 33, 4536 (1985).
22. A. McLeod, F. C. Lam, P. K. Gupta and C. T. Hung, J. Pharm. Sci., 77, 704 (1988).
23. R. Huisman, H. V. Van Kamp, J. W. Weyland, D. A. Doornbos, G. K. Bolhuis and C. F. Lerk, Pharm. Weekblad., 6, 185 (1984).
24. K. Takayama, H. Imaizumi, N. Nambu and T. Nagai, Chem. Pharm. Bull., 33, 292 (1985).

25. R. A. McLean and V. L. Anderson, Technometrics, 8, 447 (1966).
26. J. A. Cornell, "Experiments with Mixtures: Designs, Models and the Analysis of the Mixture Data", Wiley, New York, 1981.
27. T. A. Shabbir and L. Sukumar, J. Pharm. Sci., 70, 897 (1981).
28. R. J. Bellato, A. M. Dean, M. A. Moustafa, A. M. Molokhia, M. W. Gouda and T. D. Sokoloski, Int. J. Pharm., 23, 195 (1985).
29. H. Scheffe, J. Royal Stat. Soc., 20, 344 (1958).
30. R. D. Snee, J. Qual. Tech., 3, 159 (1971).
31. R. D. Snee and D. W. Marquardt, Technometrics, 16, 399 (1974).
32. C. K. Bayne and C. Y. Ma, J. Liq. Chromatogr., 10, 3529 (1987).
33. P. Y. Wang, D. W. Evans, N. Samji and E. Llewellyn-Thomas, J. Surg. Res., 28, 182 (1980).
34. SAS User's Guide, Statistics, 5th edn., North Carolina, 1985.
35. D. Queen, J. H. Evans, J. D. S. Gaylor, J. M. Courtney and W. H. Reid, Burns, 13, 218 (1987).
36. E. V. Johnston, J. S. Lundy, W. A. Bennett and J. M. Janes, Am J. Surg., 85, 720 (1953).
37. E. A. Kabat, "Structural Concepts in Immunology and Immunochemistry", 2nd ed., Holt Rinehart and Winston, New York, 1976,
38. P. M. Elias, J. Invest. Dermatol., 80 (suppl), 44s (1983).
39. G. Imokawa and M. Hattori, J. Invest. Dermatol., 84, 282 (1985).
40. G. Imokawa, H. Akasaki, M. Hattori and N. Yoshizuka, J. Invest. Dermatol., 87, 758 (1986).
41. T. M. Sweeney and D. T. Downing, J. Invest. Dermatol., 55, 135 (1970).
42. P. M. Elias, Arch. Dermatol. Res., 270, 95 (1981).
43. G. Birke, L. A. Carlson and S. O. Liljedahl, Acta Med. Scand., 178, 337 (1965).
44. C. Jelenko, M. L. Wheeler and T. H. Scott, Jr. J. Trauma, 12, 968 (1972).
45. C. Jelenko, M. L. Wheeler, A. P. Anderson, D. Callaway and R. A. Scott, J. Trauma, 75, 892 (1974).
46. C. Prottey, P. J. Hartop, J. G. Black and J. I. McCormack, Br. J. Dermatol., 94, 13 (1976).
47. C. Jelenko, W. I. Smulyan and M. L. Wheeler, Ann. Surg., 167, 521 (1968).
48. L. O. Lamke, Scand. J. Plast. Recons. Surg., 4, 1 (1970).